



Effect of Exposure to Triclosan on Reproductive System of Female Rats at Different Developmental Phases

Barnika Bandyopadhyay¹, Lakshmishri Lahiry²

¹University Research Scholar, Department of Physiology, University of Kalyani, Nadia, West Bengal-741235, India; ²Assistant Professor, Department of Physiology, University of Kalyani, Nadia, West Bengal-741235, India

ABSTRACT

Introduction: Triclosan (TCS), extensively used in different cleaning and personal care products due to its antimicrobial properties, is an endocrine disruptor that has raised health concerns.

Aim/Objective: To see the effects of exposure to sublethal dose of Triclosan on the development and function of reproductive system of female offspring rats in their early juvenile, pubertal and post pubertal stages of development.

Method: F1 generation of female rats (experimental animals), obtained from TCS treated mother rats, were exposed orally to sub-lethal doses of TCS, according to the concentration given to their respective mother, from PND 19. The body weights, reproductive organ weights, onset of puberty and estrous cycle, serum gonadotropin and ovarian hormones and histology of ovaries and uteri were studied.

Results: Pre-natal exposure to TCS through TCS-ingestion by mother rats did not affect the birth weights of the offspring. TCS treatment did not show change in the body weight gain at the end of one month when compared to untreated animals. Increased relative uterine and ovarian weights were observed upon TCS treatment. A dose dependent delay in the vaginal opening correlating with decreased serum level of FSH was observed indicating the disrupted hormonal balance that delayed the onset of puberty. TCS induced changes in number and growth of ovarian follicles and changes in thickness of uterine layers with increased width of uterine lumen was observed in all developmental phases. Prolonged TCS treatment till post pubertal stages of development induced decrease in FSH and Estradiol levels in the proliferative phases of estrous cycle and increased serum progesterone level sustained by increase in ovarian corpus luteum, thereby affecting the normal commencement of estrous cycle by increasing duration of metestrus-diestrus phases.

Conclusion: Chronic exposure of environmentally relevant concentration of TCS to female rats induced altered gonadostat resetting and delayed puberty and early onset of reproductive senescence during reproductive development indicating possibilities of infertility.

Key Words: Triclosan, Puberty, Estrous cycle, Ovaries, Uteri, Gonadotropin hormones

INTRODUCTION

Triclosan [5-chloro-2-(2,4-dichlorophenoxy) phenol] (TCS) is a chlorinated aromatic chemical compound having antimicrobial and antifungal properties. Patented in 1964, TCS has been produced and used extensively worldwide thereafter¹ in cleaning products, toothpastes, surgical sutures, sports equipment, etc., as an important disinfectant component.^{1,2} Within 14 years, from the time it got patented, it was detected as an environmental contaminant.^{1,3} Afterwards TCS has been detected in effluents and wastewater throughout the world^{4,5} including Indian waste water treatment plants⁶ and

Indian water bodies like in the rivers Vellar, Bhavani, Kaveri, Tamiraparani and Torsa etc.^{7,8} In a cohort study performed, detectable levels of TCS have been found in urine samples of all pregnant women and 51% of cord blood samples, following conjugate hydrolysis.⁹

Triclosan has also been detected in aquatic species, i.e., Indian major carp *L. Rohita* where TCS has caused genotoxicity, altered values of haematological, biochemical and histological parameters.¹⁰ Adverse effects of Triclosan have been reported by a number of *in-vitro* and *in-vivo* studies. Triclosan has been shown to damage hippocampal neuronal

Corresponding Author:

Dr. Lakshmishri Lahiry, Assistant Professor, Department of Physiology, University of Kalyani, Kalyani, Nadia, West Bengal-741235, India; Email: lakshmishrilahiry@yahoo.co.in

ISSN: 2231-2196 (Print)

ISSN: 0975-5241 (Online)

Received: 09.01.2024

Revised: 18.02.2024

Accepted: 03.03.2024

Published: 18.03.2024

function in rodent hippocampal cells¹¹ and impair the excitation-contraction coupling in murine cardiac and skeletal muscle cells.¹² Triclosan treatment resulted in reactive oxygen species generation and cell cycle arrest in human skin keratinocyte cell line¹³ whereas it increased the proliferation in cells derived from mouse epidermis.¹⁴ TCS treatment has been shown to affect reproductive system where reduced weights of testis, epididymis, prostate, seminal vesicle and vas deferens have been reported in male rats. Suppression of spermatogenesis and histological lesions in seminiferous tubules were also reported.¹⁵ Sub-cutaneous injection of TCS during gestation and lactation showed delayed testicular descent in F₁ male animals at the dose of 150 mg/Kg bodyweight. A dose dependent decrease in testosterone level, expression of steroid hormone receptors, sperm count and motility were also reported.¹⁶ Accumulation of TCS was found to be highest in the placental tissue at the end of gestation, compared to the other organs like liver, kidney in mother rat when pregnant rats were treated with dose as low as 30mg/kg/day during gestation.¹⁷ The somatic components of the ovary develop and are modified throughout postnatal folliculogenesis with significant and essential inputs from the gonadotropins, from estrogen signalling and other pathways.¹⁸ Hence, exposure to TCS which has been suggested to act as estrogenic endocrine disruptors during early in-utero development can be alarming and possess the reprogramming ability. Again, uterine functional development of rats, which is similar to *in-utero* development of human around the fourteenth week of gestation, occurs in their post-natal days.¹⁹ Therefore, we designed our experiments to see how TCS can alter the normal reproductive functions at different critical developmental endpoints of F₁ generation of female rats at environmentally relevant and sub-lethal doses where the exposures are, both direct with oral administration and indirect during *in-utero* development and throughout their respective life span.

MATERIALS AND METHODS

Experimental animals, Drugs and Dose: Male and female Sprague Dawley rats were maintained and handled in accordance with the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), India for the care and use of laboratory animals. Animals were acclimatised and maintained under controlled temperature and humidity and 12h/12h light-dark cycle with adequate supply of water and food *ad libitum*. Triclosan (CAS # 3380-34-5, lot # BCBG0479V, 97% purity), obtained from Sigma Aldrich, India, was dissolved in corn oil and administered by oral gavage to the female animals. 50 mg/kg/day dose was chosen as the highest concentration in this study with other chosen doses at 0.1%, 0.2% and 0.6% of its LD₅₀ concentration,²⁰ i.e., 5 mg, 10mg and 30mg/kg/

day. The experimental animals were F₁ generation of female rats, obtained from the F₀ generation of female who were exposed to either vehicle corn oil as control animals or administered TCS orally at 5, 10, 30 and 50 mg/kg body weight/day concentration, three weeks prior to mating. F₁ female pups or experimental animals thus obtained were weaned at their PND (post-natal day) 18 and were either untreated as their control mothers or orally administered TCS from PND 19 according to the TCS concentration given to their respective mother.

Study Design with number of subjects and groups studied

: Both TCS treated and untreated female rats were divided into three groups with each group comprising of untreated/control set and four TCS dose treated sets of animals with each set comprising of six experimental animals. The first group of animals were sacrificed on PND 22, animals of the second group sacrificed on the day of their respective vaginal opening and the third group of animals were sacrificed after two complete estrous cycles had been observed following first ovulation. Body weights of each animal were recorded on every alternate day. Beginning on the day when vaginal opening was observed, daily vaginal smears of each animal were taken followed by Hematoxylin - Eosine (HE) counter-staining and observed under microscope at 400x magnification to identify proestrous, estrous, metestrus, diestrus phases of estrous cycle.²¹

On the day of necropsy body weight of each animal was recorded and estrous phase of each experimental animal was observed. After dissection, weights of ovaries (paired) and weights of wet and blotted uteri were recorded. Blood was collected from each animal following cardiac puncture, centrifuged to isolate the serum and stored separately at -20°C until further assay.

Serum Hormone Level: To determine serum levels of Follicle Stimulating Hormone (FSH), Luteinizing hormone (LH), estradiol and progesterone the collected blood was centrifuged at 3500 rpm for 15 min to isolate the serum²² and enzyme-linked immune assay was performed using ELISA kits according to given protocols. FSH and LH were measured by sandwich ELISA method with kits obtained from Elabscience, and estradiol and progesterone were measured by competitive ELISA method with kits obtained from BTLAB, China.

Histology: Ovaries and uteri of dissected animals were fixed in Bouin's fixative, embedded in paraffin and transverse sections were cut at 5µm thickness. Six sections were taken from each ovary at 25 µm intervals in case of animals in their early juvenile and puberty and at 50 µm intervals in case of animals in estrous cycle. For uterus, six sections were taken at 100 µm intervals from both cycling and non-cycling experimental animals. Hematoxylin-Eosine (HE) counter-staining method was followed to stain the sections and im-

ages were taken with the help of a digital camera attached to the 50x magnification. Using Motic Image Plus 2.0 software thickness of uterine layers were measured. Numbers of primary, growing, antral follicles and corpus luteum in the ovarian sections were evaluated²³ using 50x magnification. Similarly the numbers of uterine endometrial stromal glands were measured.

Statistical analysis: All data were collected individually and mean and standard error of mean (SEM) were calculated. Data were assessed using a one-way ANOVA and followed by Dunnett's test. All data were compared to the respective 'control'. $P < 0.05$ was considered as the level of significance.

RESULTS

1. Effect of Triclosan on birth weight and the gain in body weight after one month of Triclosan treatment:

We have seen no significant changes in birth weight of pups (F_1 females) from TCS exposed mother animals when compared to the pups of untreated mothers (Fig. 1A). Body weight gain of the F_1 adult animals after one month of treatment showed no significant difference compared to control. (Fig. 1B)

2. Effect of brief exposure of Triclosan on development of female reproductive organs in weanlings

The weaned pups of PND18 from TCS treated mothers were exposed to similar concentration of TCS as their respective treated mothers for three days during the transition from late infantile to onset of juvenile period (PND 19 to 21) and sacrificed on PND 22. The weights of both the fluid filled (wet weight) and blotted uteri were measured (Table 1) as this is important for the assessment of any estrogenic effect induced by EDCs on uterus.^{24,25} The differences in body weights influenced by differences in weights at birth were considered a source of bias in the analysis of the organ weights²⁶ and therefore data of relative organ weights were taken. An increase of 6 to 32 % in mean relative wet weight and 7 to 32 % in mean relative blotted weight of uteri and an increase of 25 to 36.9 % in mean relative ovarian weights were observed upon treatment showing that TCS is able to alter the reproductive organ weights at even potentially lower concentration (Table 1). Our study of serum hormones (Fig. 2A) showed that by PND 22, TCS treatment at higher doses significantly decreases FSH by almost four folds the declining level of FSH after mini puberty^{27,28} without significantly changing LH levels in the treated animals. Elevated levels of estradiol were observed in dose sets as compared to untreated animals without a change in the progesterone level. The TCS-induced changes in the histology

of the ovaries (Fig. 2B) and uteri (Fig. 2C) of the early juvenile animals suggests that the number of growing follicles in ovaries (Table 2) significantly increased in TCS treated animals. The uterine luminal width of the animals increased with dose administered (Table 3). Therefore, TCS exposure affected the development of reproductive organs.

3. Effect of TCS treatment from the late infantile phase on the onset of puberty and reproductive function

To assess the effect of TCS on onset of puberty in the female offspring of TCS treated F_0 female animals, TCS was administered orally according to the concentration given to their mothers from the end of late infantile period (PND 19) throughout the juvenile stage till the day of onset of puberty marked by vaginal opening in these animals. A significant mean delay of 10 to 22 days in vaginal opening was observed in animals treated with doses of 10mg, 30 mg and 50mg TCS/kg/day when compared to untreated animals (Fig. 3A). During pubertal development the ovarian weight as well as weights of uteri increase with increase in the number of fluid filled ovulatory follicle and the change in histo-architecture of uteri from its quiescent state respectively.^{29, 30, 31} At this developmental phase, the relative uterine wet and blotted weights increased in 50 mg/kg/day treated animals as compared to untreated set. The relative ovarian weights increased in all TCS treated animals (Table 4). The transverse sections of ovary showed a significant increase of up to two-fold in mean number of growing follicles in 30mg and 50mg/kg/day TCS dose-treated animals without significant differences in the numbers of primary and antral follicles compared to untreated ones (Table 5). Appearance of corpus luteum in ovarian sections were observed in these pubertal animals (Fig. 3B), without any significant change in their numbers upon TCS treatment (Table 5). Hematoxylin-eosin stained section of uteri (Fig. 3C) showed that width of uterine lumen increased significantly in treated animals with maximum increase of 2.34 folds in the highest dose treated animals (Table 6). A significant increase in the thickness of uterine perimetrium, increased thickness of myometrium and endometrial stroma were observed in TCS treated rat uteri when compared to uteri of control animals (Table 6). The height of luminal epithelial layer of uteri increased with TCS treatment although the numbers of uterine glands decreased upon 5mg and 10mg/kg/day TCS treatment when compared to control animals (Table 6). The maturation of HPG axis completes when the estradiol mediated pre-ovulatory LH and FSH surge leads to the first ovulation with a shift towards increasing level of progesterone after ovulation.^{31, 32} With treatment of Triclosan, the level of serum FSH decreased by 2.89 folds in comparison to untreated animals at the time of onset of puberty whereas the serum level of LH increased but not significantly (Fig. 3D). The serum levels of

gonadal hormones did not alter significantly upon treatment with different doses of TCS in comparison with untreated set on the respective day of vaginal opening (Fig. 3D).

4. Effect of TCS on commencement of reproductive cycle in post pubertal animals.

The post pubertal reproductive development in female rats is a critical window between the opening of vagina and establishment of regularity in hormonal cycle and estrous cycle that is influenced by endocrine disruptor chemical.³³ To study the effect of the drug at this developmental phase, female offspring of TCS treated F_0 animals were weaned at PND 18 and were orally administered TCS at concentrations given to their mothers, from the end of late infantile period (PND 19) till the dissection of the animals at each of the different phases of estrous cycle following completion of first two estrous cycles. The estrous cycle in untreated animals was found to be a normal four day cycle. As our result shows that vaginal opening in treated animals was significantly delayed with higher concentration of dose when compared to the untreated animals, a significant delay of up to eleven days to complete two estrous cycles from the onset of puberty was observed in treated animals as compared to the eight days taken by untreated animals to complete the first two estrous cycles (Fig. 4A). Therefore, the age of the animals after completion of two estrous cycles from the onset of puberty significantly delayed from PND 45 in control to PND 57 - 70 in 10, 30 and 50 mg/kg/day dose treated animals (Fig. 4B). Figure 4C shows the record of first two estrous cycles of one representative animal from each dose set and the control. The number of days spent in each estrous phase in the first two complete cycles by animals of each treatment sets (Fig. 4D) shows that the duration of metestrus- diestrus phases were prolonged in all treated animals with a significant extension of up to five days in animals ingesting highest concentration of TCS.

To exclude the effects of changes that occur during different phases of estrous cycle on the reproductive organ weights, we took the uterine and ovarian weights of the female rats in their diestrus phases to elucidate the effect of TCS treatment.³² TCS treated animals of F_1 generation, in post pubertal phase of life had showed a significant increase in the relative uterine wet and blotted weights in 30 mg and 50 mg/kg/day treatment sets as compared to untreated animals. Triclosan treatment increased the weights of ovary and higher relative ovarian weights were observed when compared to animals without TCS treatment (Table 7). After first two estrous cycles, ovaries of TCS treated animals had similar numbers of primary, growing and antral follicles and a significantly higher number of corpus luteum in 10mg and 30mg/kg/day treatment sets in comparison with control animals (Table 8). After the first ovulation and first estrous cycle, the uteri of female rats normally achieve a diameter

of approximately 1.75 mm.³⁰ Histology of uteri, after first two complete estrous cycles, showed a wider uterus with a maximum diameter of 2.21mm in higher dose-treated animals with a corresponding significant increase in thickness of myometrium and increased height of luminal epithelium of uteri at diestrus phase (Table 9, Fig 4E and 4F). The serum levels of FSH, LH, estradiol and progesterone of the animals were measured in each of the different estrous phases (Fig. 4G). In 30 mg and 50 mg/kg/day TCS treated rats the FSH level was significantly low in diestrus and proestrous phases where the maximum decrease was 2.5-fold in comparison with control. The level of LH significantly increased in proestrous phase of 5mg TCS/kg/day treated rats and in estrous phase of 30mg/kg/day treated animals when compared to untreated animals. A significant decrease in serum FSH level corresponds to decrease in estradiol level in animals in their diestrus phases of all dose sets and in proestrous and estrous phases of higher dose-treated animals when compared to control. An increase in the number of corpus luteum in TCS-treated ovary increased the level of progesterone synthesis not only in estrous and metestrus phases of all dose-treated animals but also serum progesterone level remained high in diestrus and proestrous phases of higher dose treated animals when compared to untreated animals.

DISCUSSION

The aim of this study is to find how the chronic TCS exposure, at low doses, affects the reproductive functions at critical phases of development of F_1 generation of female pups from TCS exposed mothers. In most of the traditional risk assessment studies of toxic substances, the harmful effects are seen in higher doses but EDCs do not follow a linear dose response curve and the adverse effects may also be seen in lower doses.³⁴ Endocrine disruptors are known to affect the intra-uterine growth which affects the birth-weights of the animals.³⁵ Our study showed no change in the birth weights of female pups of TCS exposed mothers and TCS treatment did not significantly change body weight gain at the end of one month.

From early juvenile phase, the brief exposure to oral doses of TCS in weanlings of mothers exposed to TCS showed the onset of changes in gonadotropin level in the serum with decreasing serum FSH level and a concomitant increase in estradiol. In rats, normally a 'mini puberty' of infancy is observed where the serum FSH increases from PND 10 to PND 14 and then decrease from PND 17.^{27,28} The Estradiol, which is freely available by the late infantile stage gives negative feedback to gonadotropins³¹ thereby decreasing the FSH level as observed upon TCS treatment. At higher dose TCS mediated augmented estrogen increased the number of ovarian growing follicles in late infantile animals and at potentially low doses, TCS can influence the increase in

ovarian weights. TCS changed uterine histology with wider uterine lumen observed in all treated animals and increased uterine weight under the influence of estrogen like action.³⁶ Therefore, TCS imposed its adverse effects even at potentially low doses at late infantile stage of development by creating a condition of hypogonadotropic-hypergonadism³⁷ with primary ovarian hyper-function and over-production of estradiol in treated female rats.

The onset of puberty marked by vaginal opening following first ovulation in rats²⁹ is directed by complex and orchestrated function of hypothalamo-pituitary-gonadal axis and preparation for this event starts from juvenile phase^{38, 31} of development. Sensitivity of hypothalamic gonadostat to ovarian steroidal negative feedback decreases as the animal matures resulting in gradual increase in serum gonadotropin, and production of more estradiol resulting in the process of gonadostat resetting.³² During peri-pubertal development, the estrogen primed female rats show increased bimodal secretion of LH characterised by afternoon surge that is facilitated by progesterone.^{39, 32} Our study showed TCS induced significant delay in vaginal opening and onset of puberty in treated rats. We can interpret that this delay could be due to longer time taken for gonadostat resetting as a consequence of TCS mediated disruption of hormonal changes that inhibit rise and stimulation of gonadotropin levels in peri-pubertal phase in the background of an already increased serum estradiol present in early juvenile phase. Our results show that although the serum FSH decreased as a result of TCS treatment, the serum level of LH and gonadal hormones did not alter with TCS treatment when observed at their respective first estrous. From our observation we found an increase in the number of ovarian growing follicles upon TCS treatment without an increase in the number of antral follicles. At the time of juvenile development, the uteri remain quiescent with absence of intrauterine fluid till peri-pubertal development, after which, at first proestrous, a 'ballooned' appearance of uteri is evident caused by fluid accumulation in the luminal space and the fluid is again almost absent just after the first ovulation takes place at first estrous.²⁹ An increase in relative uterine weight was observed in animals treated with higher concentrations of TCS with an increase in uterine luminal width, increased thickness of uterine layers which could be correlated with TCS-induced prolonged estrogenic stimulation at a concentration insufficient to cause ovulation. However the numbers of uterine endometrial stromal glands were decreased in rats administered with lower doses of TCS.

Hence it can be concluded that TCS treatment till puberty interrupted gonadostat resetting, altered endogenous gonadal hormones and delayed the first ovulation. After vaginal opening, it was observed that more number of days were required to complete the first two estrous cycles in animals treated with higher doses of TCS that also parallely showed delayed onset of puberty as compared to control. The prolonged me-

testrous-diestrous phase as observed in TCS treated animals reflected upon the progesterone-driven state of the reproductive organs which was evident with increased ovarian corpus luteum and concomitant increase in progesterone level in estrous and metestrus phases of higher dose treated sets of animals. The decreased level of serum FSH and estradiol in diestrus and proestrous phases of higher dose treated animals implied the slower follicular maturation which led to lesser number of estrous cycle in a given span compared to control. However lower serum estradiol level across the estrous phases did not reciprocate the increased sensitivity of uterine layers of TCS treated animals to these hormones. The significant increase in uterine weight and thickness of uterine myometrium and endometrial epithelium at the time of the initiation and first few reproductive cycles indicates the possibility that TCS may induce leiomyoma in adulthood.⁴⁰ From our study the effect of TCS, passed on by drug exposed mother rats to their female pups, who were further administered TCS during their development, highlighted the issues of critical window of susceptibility to potential reproductive health effects of TCS during their early life.

CONCLUSION

As we are exposed to EDCs like Triclosan in our daily life, studies with chronic exposure to environmentally relevant concentration of TCS should receive more attention beside traditional risk assessment with acute doses. Our results suggest chronic exposure to sublethal dose of TCS affected reproductive development in female rats inducing reproductive disorders and thereby indicating adverse effect on fertility. Further this study is unique where offspring rats are indirectly exposed to TCS during intra-uterine development and lactation period apart from the direct oral ingestion of TCS during infantile and pubertal development showing how continuous exposure augment disruptive effects of TCS on female reproductive system.

ACKNOWLEDGEMENT

The authors acknowledge the help received from Dr. Neera Sen Sarkar, Dept. of Botany, and S. N. Bose Innovation Centre (Instrumentation), University of Kalyani for providing bright field microscope with image capturing facility and Motic Images Plus Software.

Source of Funding- This research did not receive any extramural research funding and was supported by personal research grant and DST PURSE II grant received by the authors from University of Kalyani.

Conflict of interest: None

Authors' Contribution:

Barnika Bandyopadhyay- Experimentation, Data Collection and analysis, writing of the study

Dr. Lakshmishri Lahiry- Conception, analysis, write up review & submission

REFERENCES

- Halden RU. On the Need and Speed of Regulating Triclosan and Triclocarban in the United States. *Environ Sci Technol*. 2014; 48:3603-3611.
- Food and Drug Administration of United States. National Toxicology Program, Department of Health and Human Services, Nomination Profile, Triclosan [CAS3380-34-5], Supporting Information for Toxicological Evaluation Toxicology Program. 2008.
- Blair BD, Crago JP, Hedman CJ, Klaper RD. Pharmaceuticals and personal care products found in the Great Lakes above concentrations of environmental concern. *Chemosphere*. 2013; 93:2116–2123.
- Jungclaus G, Avila V, Hites R. Organic compounds in an industrial wastewater: A case study of their environmental impact. *Environ. Sci. Technol*. 1978; 12:88-96
- Cheng CY, Wang YC, Ding WH. Determination of Triclosan in aqueous samples using solid-phase extraction followed by on-line derivatization gas chromatography-mass spectrometry. *Anal. Sci*. 2011; 27:197–202
- Balakrishna K, Rath A, Praveenkumar Reddy Y, Guruge KS, Subedi B. A review of the occurrence of pharmaceuticals and personal care products in Indian water bodies. *Ecotoxicol. Environ. Saf*. 2017; 137:113–120
- Ramaswamy BR, Shanmugam G, Velu G, Rengarajan B, Larsen DGJ. GC–MS analysis and ecotoxicological risk assessment of triclosan, carbamazepine and parabens in Indian rivers. *J Hazard Mater* 2011; 186:1586–1593
- Sarkar SD, Nag SK, Kumari K, Saha K, Bandyopadhyay S, Aftabuddin M, et al. Occurrence and safety evaluation of antimicrobial compounds Triclosan and Triclocarban in Water and Fishes of the Multitrophic Niche of River Torsa, India. *Arch. Environ. Contam. Toxicol*. 2020; 79:488–499
- Pycke BF, Geer LA, Dalloul M, Abulafia O, Jenck AM, Halden RU. Human fetal exposure to Triclosan and Triclocarban in an urban population from Brooklyn, New York. *Environ. Sci. Technol*. 2014; 48: 8831-8838
- Hemalatha D, Nataraj B, Rangasamy B, Shobana C, Ramesh M. DNA damage and physiological responses in an Indian major carp *Labeorohita* exposed to an antimicrobial agent Triclosan. *Fish Physiol Biochem*. 2019;45(4):1463-1484
- Arias-Cavieres A, More J, Vicente JM, Adasme T, Hidalgo J, Valdés JL, et al. Triclosan impairs hippocampal synaptic plasticity and spatial memory in male rats. *Front Mol Neurosci*. 2018; 11:429
- Cherednichenko G, Zhang R, Bannister RA, Timofeyev V, Li N, Fritsch EB, et al. Triclosan impairs excitation-contraction coupling and Ca²⁺ dynamics in striated muscle. *Proc Natl Acad Sci USA*. 2012; 109 (35):14158–14163
- Dubey D, Srivastav AK, Singh J, Chopra D, Qureshi S, Kushwaha HN, et al. Photoexcited triclosan induced DNA damage and oxidative stress via p38 MAP kinase signalling involving type I radicals under sunlight/UVB exposure. *Ecotoxicol Environ Saf*. 2019; 15(174):270-282
- Wu Y, Beland FA, Chen S, Fang JL. Extracellular signal-regulated kinases 1/2 and Akt contribute to Triclosan-stimulated proliferation of JB6 Cl 41–5a cells. *Arch Toxicol*. 2015; 89(8):1297–1311
- Kumar V, Chakraborty A, Kural M R, Roy P. Alteration of testicular steroidogenesis and histopathology of reproductive system in male rats treated with Triclosan. *Reprod Toxicol*. 2009; 27:177-185
- Priyanka, Trivedi A, Maske P, Mote C, Dighe V. Gestational and lactational exposure to Triclosan causes impaired fertility of F1 male offspring and developmental defects in F2 generation. *Environ Pollut*. 2020; 257:113617.
- Feng Y, Zhang P, Zhang Z, Shi J, Jiao Z, Shao B. Endocrine Disrupting Effects of Triclosan on the Placenta in Pregnant Rats. *PLoS ONE*. 2016; 11(5): e0154758.
- Richards JS, Sharma SC, Falender AE, Lo YH. Expression of FKHR, FKHRL1, and AFX genes in the rodent ovary: evidence for regulation by IGF-I, estrogen, and the gonadotropins. *Mol Endocrinol*. 2002;16(3):580-599
- Zama AM, Bhurke A, Uzumcu M. Effects of Endocrine-disrupting chemicals on female reproductive health. *The Open Biotechnol J*. 2016; 10(Suppl-1, M6):54-75
- Bhargava HN, Leonard PA. Triclosan: applications and safety. *Am J Infect Control*. 1996;24:209-218
- Goldman JM, Murr AS, Cooper RL. The rodent estrous cycle: Characterization of vaginal cytology and its utility in toxicological studies. *Birth defects Res B Dev Reprod Toxicol*. 2007; 80:84-97
- Wisniewski P, Romano RM, Kizys MM, et al. Adult exposure to bisphenol A (BPA) in Wistar rats reduces sperm quality with disruption of the hypothalamic-pituitary-testicular axis. *Toxicology*. 2015;329:1-9
- Pedersen T, Peters, H. Proposal for a classification of oocytes and follicles in the mouse ovary. *J Reprod Fertil*. 1968; 17: 555–557
- Goldman JM, Laws SC, Balchak SK, Cooper RL, Kavlock RJ. Endocrine-disrupting chemicals: prepubertal exposures and effects on sexual maturation and thyroid activity in the female rat. A focus on the EDSTAC recommendations. *Crit Rev Toxicol*. 2000; 30(2):135-196
- Kwekel JC, Burgoon LD, Burt JW, Harkema JR, Zacharewski TR. A cross-species analysis of the rodent uterotrophic program: elucidation of conserved responses and targets of estrogen signalling. *Physiol Genomics*. 2005; 23:327-342.
- Piao Y, Liu Y, Xie X. Change trends of organ weight background data in sprague-dawley rats at different ages. *J Toxicol Pathol*. 2013;26(1):29-34.
- François CM, Petit F, Giton F, Gougeon A, Ravel C, Magre S et al. A novel action of follicle-stimulating hormone in the ovary promotes estradiol production without inducing excessive follicular growth before puberty. *Sci Rep*. 2017; 7:46222.
- Döhler KD, Wuttke W. Serum LH, FSH, prolactin and progesterone from birth to puberty in female and male rats. *Endocrinology*. 1974; 94(4):1003-1008
- Ojeda SR., Skinner MK. Puberty in the rat. In: Neill JD, Plant TM, Pfaff DW, Challis JRG, de Kretser DM et al., editors. *Knobil and Neill's Physiology of Reproduction*. 3 ed. Vol. 2. San Diego, CA ; Academic Press: 2006. p. 2061-2126
- Picut CA, Remick AK, Asakawa MG, Simons ML, Parker GA. Histologic features of prepubertal and pubertal reproductive development in female Sprague-Dawley rats. *Toxicol Pathol*. 2013; 42(2):403-413

31. Picut CA, Dixon D, Simons ML, Stump DG, Parker GA, Remick AK. Postnatal ovary development in the rat: morphologic study and correlation of morphology to neuroendocrine parameters. *Toxicol Pathol.* 2015;43(3):343-353
32. Ojeda SR, Andrews WW, Advis JP, White SS. Recent advances in the endocrinology of puberty. *Endocr Rev.* 1980; 1(3):228-257
33. Scsukova S, Rollerova E, Bujnakova Mlynarcikova A. Impact of endocrine disrupting chemicals on onset and development of female reproductive disorders and hormone related cancer. *Reprod Biol.* 2016; 16(4):243-254
34. Lee D, Jacobs DR. New approaches to cope with possible harms of low-dose environmental chemicals. *J Epidemiol Community Health* 2019;73:193-197.
35. EPA-US Guidelines for reproductive toxicity risk assessment, Federal Register, 1996; 61 (212): 56274-56322
36. Shelby MD, Newbold RR, Tully DB, Chae K, Davis VL. Assessing environmental chemicals for estrogenicity using a combination of in vitro and in vivo assays. *Environ Health Perspect.* 1996;104(12):1296-1300
37. Cook JD. Reproductive endocrinology in infertility. *Lab. Med.* 2004; 35(9): 558-569
38. A M Brown, J M Janik, E S Murphree, R King, P Callahan. Effects of cyclic steroid hormone replacement on prolactin and luteinizing hormone surges in female rats. *Reproduction* 2004; 128:373–378.
39. Caligaris L, Astrada J, Talesnik S. Influence of age on the release of luteinizing hormone induced by estrogen and progesterone in immature rats. *J Endocrinol* 1972. 55:97
40. Crain DA, Janssen SJ, Edwards TM, Heindel J, Ho S, Hunt P et al. Female reproductive disorders: the roles of endocrine disrupting compounds and developmental timing. *Fertil Steril.* 2008; 90(4):911-940

Table 1: Mean absolute and relative weights of ovaries and uteri of untreated and TCS treated female offspring in early juvenile phase.

	Control	5 mg	10 mg	30 mg	50 mg
Body weight on dissection day (gm)	29.10±0.24	24.16±0.53	26.27±0.34	21.66±0.49	28.13±0.62
Uterine wet weight (gm)	0.030±0.0002	0.033±0.0004	0.032±0.001	0.028±0.0002	0.031±0.001
Uterine blotted weight (gm)	0.029±0.0001	0.031±0.0003	0.030±0.0004	0.026±0.0002	0.029±0.001
Relative uterine wet weight	0.103±0.001	0.136±0.002*	0.123±0.001*	0.131±0.003*	0.110±0.001*
Relative uterine blotted weight	0.098±0.001	0.130±0.001*	0.114±0.001*	0.120±0.003*	0.105±0.001*
Ovarian weight (gm)	0.032±0.0002	0.034±0.001	0.029±0.0004	0.033±0.0005	0.032±0.001
Relative ovarian weight	0.111±0.001	0.139±0.002*	0.111±0.001	0.152±0.004*	0.112±0.001

Values are expressed as the Mean ± SEM (n=6 animals/ set), analysed by ANOVA, *P<0.05, and followed by Dunnett's test only in case of relative ovarian and uterine weights, where values are compared to control.

Table 2: Mean count of primary, growing and antral follicles of ovaries from untreated and TCS treated female offspring of early juvenile phase.

Dose Group	Primary follicles	Growing follicles	Antral follicles
Control	10.76±0.63	8.88±0.53	3.35±0.16
5 mg	10.43±0.33	11.93±0.70*	4.67±0.15
10 mg	10.11±0.28	14.10±0.35*	3.71±0.21
30 mg	9.97±0.63	12.31±1.10*	4.19±0.15
50 mg	9.54±0.41	12.11±0.47*	3.92±0.34

Values are expressed as the Mean ± SEM (n=6 animals/ set), analysed by ANOVA, *P<0.05, followed by Dunnett's test; all values are compared to control.

Table 3: Uterine lumen width and thickness of different layers of uterus from untreated and TCS treated female offspring in early juvenile phase.

Dose group	Uterine lumen width (µm)	Thickness of uterine layers (µm)			
		Perimetrium	Myometrium	Endometrial stroma	Luminal epithelium
Control	67.38±2.04	13.72±0.48	77.16±8.72	156.36±8.47	10.95±0.75
5 mg	106.81±7.38*	19.77±1.09	72.97±5.56	153.10±8.01	14.39±1.24
10 mg	124.37±2.14*	19.19±3.01	57.36±1.49	133.43±3.33	12.78±0.78
30 mg	127.57±10.81*	16.82±0.52	66.70±1.44	156.84±1.85	12.84±0.53
50 mg	105.61±6.39*	14.54±0.93	90.94±6.78	165.31±2.87	12.97±1.02

Values are expressed as the Mean ± SEM (n=6) and analysed by ANOVA, *P<0.05, followed by Dunnett's test; all values are compared to control.

Table 4: Weights of reproductive organs (absolute and relative to body weights) of untreated and TCS treated F₁ female rats at puberty

	C	5 mg	10 mg	30 mg	50 mg
Body weight on dissection day (gm)	54.31±4.66	52.81±1.96	49.02±2.90	60.41±1.83	70.56±2.30
Uterine wet weight (gm)	0.053±0.007	0.057±0.003	0.059±0.001	0.074±0.007	0.129±0.030
Uterine blotted weight (gm)	0.044±0.007	0.053±0.003	0.054±0.001	0.068±0.007	0.115±0.027
Relative uterine wet weight	0.096±0.005	0.109±0.006	0.121±0.005	0.121±0.008	0.181±0.037*
Relative uterine blotted weight	0.078±0.006	0.101±0.004	0.112±0.004	0.112±0.007	0.161±0.034*
Ovarian weight (gm)	0.040±0.002	0.057±0.003	0.052±0.004	0.063±0.001	0.066±0.006
Relative ovarian weight	0.076±0.003	0.108±0.001*	0.105±0.002*	0.104±0.002*	0.093±0.006*

Values are expressed as the Mean ± SEM (n=6 animals/ set), analysed by ANOVA, *P<0.05, followed by Dunnett's test only in case of relative ovarian and uterine weights, where only in case of relative ovarian and uterine weights, values are compared to control 'C'.

Table 5: Differential count of follicles and corpus luteum of ovaries from untreated and TCS treated F₁ female rats at puberty.

Dose Group	Primary follicles	Growing follicles	Antral follicles	Corpus luteum
Control	4.83±0.93	4.83±0.96	4.17±0.44	1.17±0.36
5 mg	4.50±0.94	6.17±0.93	5.67±0.56	1.50±0.20
10 mg	4.67±1.01	5.50±0.84	4.50±0.91	0.83±0.28
30 mg	3.67±0.73	9.00±0.97*	8.33±0.56	1.00±0.33
50 mg	4.17±0.80	11.33±0.81*	3.67±0.84	1.33±0.45

Values are expressed as the Mean ± SEM (n=6 animals/ set, analysed by ANOVA, *P<0.05, followed by Dunnett's test; all values are compared to control.

Table 6: Width of lumen, thickness of different layers and numbers of endometrial glands of uteri from untreated and TCS treated F₁ female rats at puberty.

Dose group	Uterine lumen width (µm)	Thickness of uterine layers (µm)				Number of uterine endometrial glands
		Perimetrium	Myometrium	Endometrial stroma	Luminal epithelium	
Control	89.51±6.36	23.42±0.80	74.32±2.75	149.69±12.11	16.08±1.36	13.67±0.69
5 mg	115.88±9.63*	29.15±2.45	73.66±4.64	176.82±15.13	16.69±1.07	9.67±0.65*
10 mg	104.22±1.23	19.54±1.54	80.43±6.81	233.14±7.12	19.24±0.92	9.50±0.91*
30 mg	175.84±28.08*	42.41±4.82*	79.89±8.39	258.48±47.11	18.75±3.72	10.83±0.83
50 mg	209.64±7.23*	45.61±6.14*	151.94±25.19	308.41±52.52	27.29±4.18	10.50±0.70

Values are expressed as the Mean ± SEM (n=6 animals/ set, analysed by ANOVA, *P<0.05, followed by Dunnett's test; all values are compared to control.

Table 7: Mean absolute and relative weights of ovaries and uteri of untreated and TCS exposed F₁ female rats at post puberty after completion of first two estrous cycle.

	Control	5 mg	10 mg	30 mg	50 mg
Body weight on dissection day (gm)	73.38±2.03	68.88±2.27	90.88±1.64	93.89±4.35	108.59±1.67
Uterine wet weight (gm)	0.209±0.005	0.200±0.01	0.218±0.001	0.340±0.024	0.420±0.009
Uterine blotted weight (gm)	0.202±0.005	0.191±0.01	0.201±0.002	0.316±0.019	0.386±0.013
Relative uterine wet weight	0.285±0.003	0.291±0.01	0.240±0.005	0.363±0.020*	0.387±0.007*
Relative uterine blotted weight	0.276±0.003	0.277±0.013	0.222±0.004*	0.338±0.017*	0.356±0.011*
Ovarian weight (gm)	0.052±0.002	0.090±0.005	0.084±0.001	0.104±0.004	0.107±0.004
Relative ovarian weight	0.070±0.002	0.132±0.011*	0.093±0.002*	0.112±0.004*	0.098±0.003*

Values are expressed as the Mean ± SEM (n=6 animals/ set, analysed by ANOVA, *P<0.05, and followed by Dunnett's test only in case of relative uterine and ovarian weights, where values are compared to control.

Table 8: Ovarian follicle quantification and count of corpus luteum of ovaries from untreated and TCS treated F₁ female rats at post puberty after completion of first two estrous cycle at their diestrus phase of cycle.

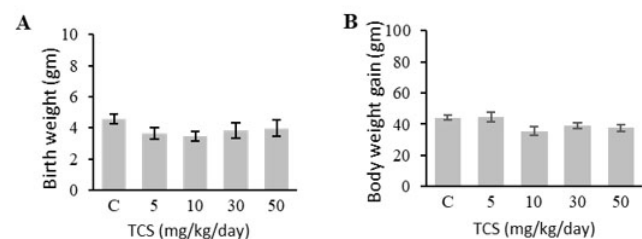
Dose Group	Primary follicles	Growing follicles	Antral follicles	Corpus Luteum
Control	3.83±0.64	5.17±0.68	3.50±0.57	3.33±0.45
5 mg	5.66±0.56	6.67±0.90	4.16±1.04	7.16±0.80
10 mg	5.67±1.14	4.66±0.61	3.17±0.80	10.16±0.72*
30 mg	6.83±0.96	5.16±1.16	3.66±0.90	7.83±1.06*
50 mg	7.16±1.01	5.67±1.02	3.33±0.93	5.33±0.96

Values are expressed as the Mean ± SEM (n=6 animals/ set, analysed by ANOVA, followed by Dunnett's test, *P<0.05, all values are compared to control)

Table 9: Morphometric analysis of uterine lumen, uterine layers and count of endometrial glands in uteri from untreated and TCS treated post pubertal F₁ female offspring rats at their diestrus phase after the first two estrous cycle.

Dose group	Uterine lumen width (µm)	Thickness of uterine layers (µm)				Number of uterine endometrial glands
		Perimetrium	Myometrium	Endometrial stroma	Luminal epithelium	
Control	158.63±4.74	77.26±3.39	108.87±5.39	472.68±3.22	15.65±1.38	10.5±0.39
5 mg	196.46±9.09	93.04±3.25	173.85±6.35*	489.06±12.87	26.64±2.58	9.33±1.24
10 mg	189.08±10.07	74.04±3.33	139.46±3.37	523.13±8.38	29.20±1.45*	7.5±0.70
30 mg	214.31±11.02	89.58±5.31	161.67±9.60*	474.38±13.70	35.31±3.73*	7.33±0.77
50 mg	527.99±12.68*	86.91±6.99	195.14±12.67*	528.96±23.02	35.67±3.76*	11.83±0.86

Values are expressed as the Mean ± SEM (n=6 animals/ set, analysed by ANOVA, *P<0.05, followed by Dunnett's test; all values are compared to control)

**Figure 1:** A. Birth weight of untreated 'C' and TCS treated F₁ female rats of untreated and dose exposed mother rats respectively; B. Body weight gain of untreated 'C' and TCS treated F₁ rats after one-month of dose exposure. Values are expressed as the Mean ± SEM (n=6 animals/ set), analysed by ANOVA, P>0.05; all values are compared to control 'C'.

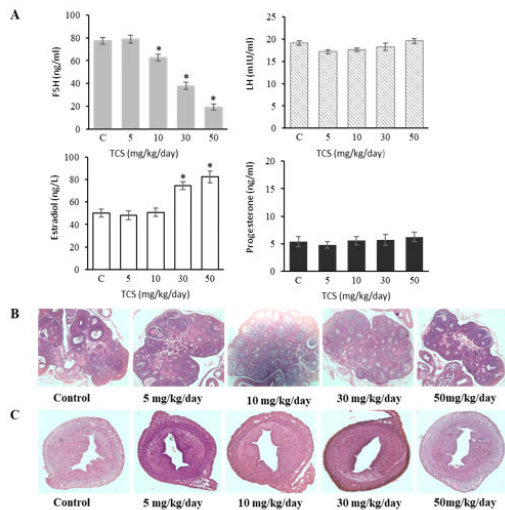


Figure 2: A. Serum level of FSH, LH, estradiol and progesterone hormones of untreated 'C' and TCS treated F female rats at early juvenile phase of development. Each bar represents the Mean $\pm$ SEM of six animals analysed by ANOVA, *P<0.05, followed by Dunnett's test where values are compared to control. B,C. Representative images of H-E-stained cross sections of ovary(B)and uterus(C)of untreated 'Control' and TCS treated early juvenile female rats. Images taken are at 50x magnification.

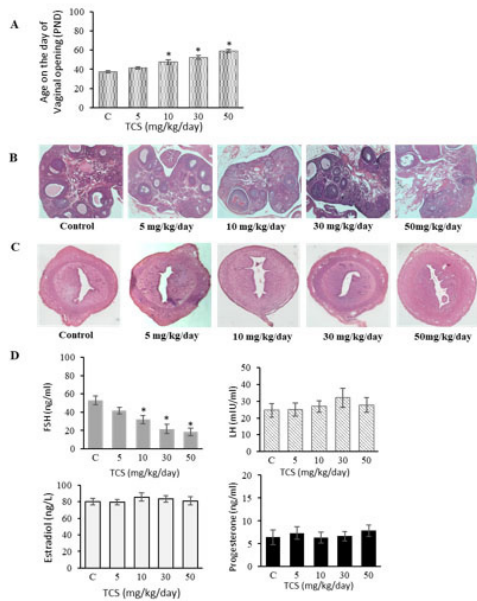


Figure 3: A. Bar diagram represents post-natal days on which vaginal opening of the untreated and treated pubertal female rats were observed. Values are expressed as the Mean $\pm$ SEM of 6 animals, analysed by ANOVA, followed by Dunnett's test, *P<0.05, all values are compared to control 'C'. B, C. Representative images (50x magnification) of H-E-stained cross sections of ovary (B) and uterus (C) of untreated and treated pubertal female rats. D. Serum level of gonadotropin hormones FSH and LH, and gonadal estradiol and progesterone of the untreated and treated pubertal female rats. Values are expressed as the Mean $\pm$ SEM (n=6 animals/ set, analysed by ANOVA, followed by Dunnett's test, *P<0.05, all values are compared to control 'C'.

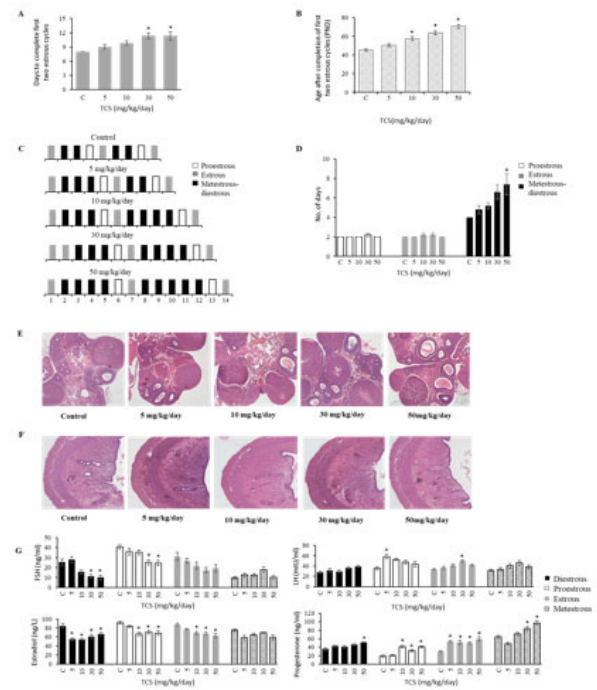


Figure 4: A. Number of days taken by untreated and treated post pubertal female rats to complete first two estrous cycles. Each bar represents values expressed as Mean $\pm$ SEM (n=6 animals/ set, analysed by ANOVA, followed by Dunnett's test, *P<0.05, all values are compared to control 'C'. B. Bar diagram represents age (in postnatal days, PND) of untreated and treated post pubertal female rats after completion of two estrous cycles where 'estrous' phase was considered as the endpoints of the cycles. Values are expressed as the Mean $\pm$ SEM of 6 animals/ set, analysed by ANOVA, followed by Dunnett's test, *P<0.05, all values are compared to control, 'C'. C. Record of first two complete estrous cycles of representative untreated 'C' and TCS treated post pubertal female rats, D. Number of days to complete proestrous phases, estrous phases and metestrous-diestrous phases by untreated 'C' and TCS-treated post pubertal female rats during the first two estrous cycles. Values are expressed as the Mean $\pm$ SEM (n=6 animals/ set, analysed by ANOVA, followed by Dunnett's test, *P<0.05, all values are compared to control of respective estrous cycle phases). E, F. Representative images at 50x magnification, of H-E-stained cross sections of ovary (E) and uteri (F) of control and treated post pubertal female rats at their diestrus phase. G. Serum level of FSH, LH, estradiol and progesterone of the untreated 'C' and treated post pubertal female rats in diestrus, proestrous, estrous and metestrous phases of estrous cycle. Values are expressed as the Mean $\pm$ SEM of 3 animals in each phase of estrous cycle per dose set, analysed by ANOVA, followed by Dunnett's test, *P<0.05, all values are compared to control 'C' of respective phases of estrous cycle).